American Fern Journal 93(3):137–145 (2003)

Rapid Gametophyte Maturation in *Ophioglossum crotalophoroides*

DEAN P. WHITTIER
Department of Biological Sciences, Vanderbilt University, Nashville, TN 37235-1565

ABSTRACT.—With most species of the Ophioglossaceae, gametophyte development and maturation are slow and some species have perennial gametophytes. A few species, including *O. crotalophoroides*, appear to have gametophytes that mature rapidly. To determine how fast the gametophytes of this species mature, they were grown in axenic culture. The early sequence of cell divisions following germination is the same as for other species of the Ophioglossaceae. The formation of mucilage on the proximal cell of the young gametophyte and on the rhizoids of older gametophytes has also been reported for other members of the family. The spores of *O. crotalophoroides* have the second fastest germination, 8 days, for this family. Gametophytes of this species grow faster than gametophytes of two *Botrychium* species. The gametangia form on smaller gametophytes of *O. crotalophoroides* than on those of *Botrychium*. Rapid spore germination, rapid gametophyte growth, and smaller gametophyte size at maturity all contribute to the formation of sexually mature gametophytes in 6.5 months. This is the fastest gametophyte maturation reported for the family.

Gametophyte development in the Ophioglossaceae is sluggish (Boullard, 1963). The spores typically take a long time to germinate (Raghavan, 1989) and the growth of the gametophyte after germination is slow (Nayar and Kaur, 1971). Some species have perennial gametophytes (Campbell, 1911; Pant *et al.*, 1984) and it can be a matter of years before sexual reproduction occurs (Bruchmann, 1904). Culturing gametophytes of the Ophioglossaceae under axenic culture conditions does not appear to accelerate their development because it took 22 months for gametophytes of *Botrychium dissectum* Spreng. to become sexually mature (Whittier, 1972).

Although gametophyte development in a majority of the species in this family takes a long time, a few species appear to mature more rapidly. Campbell (1907) concluded that *Ophioglossum moluccanum* Schlect had annual gametophytes. Gametophytes of *Helminthostachys zeylanica* (L.) Hook. and *Ophioglossum nudicaule* L. are reported to be short lived by Nozu (1961) and Mesler *et al.* (1975) respectively. It also appears that *Ophioglossum crotalophoroides* Walt. has rapid gametophyte development because Mesler (1976) found mature gametophytes one year after spores were released into pots under greenhouse conditions.

What causes accelerated gametophyte maturation in some species of the Ophioglossaceae has never been examined. A study on gametophyte development in *O. crotalophoroides* presented an opportunity to examine rapid maturation in this group. This investigation was carried out to determine how fast gametophytes of this species become sexually mature and, if possible, what accelerates gametophyte maturation.

Materials and Methods

Spores of *Ophioglossum crotalophoroides* were obtained from plants in Alabama and Louisiana. Vouchers of the sporophytes are on deposit at the Vanderbilt University Herbarium (VDB). The spores were usually sown within a month of their collection. To reduce the incidence of contamination, the spores were wetted and stored in water for 24 hours before surface sterilization. They then were surface sterilized with 20% Clorox (1.1% sodium hypochlorite) by the method of Whittier (1964), collected on sterile filter paper, suspended in sterile water, and sown on 15 ml of nutrient medium in culture tubes (20 × 125 mm) with screw caps that were tightened to reduce moisture loss. Most of the cultures were maintained at 21 ± 1°C in the dark, but a few had exposures to light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$) from Gro-lux fluorescent lamps for 14 of every 24 hours.

The basic nutrient medium contained 100 mg $MgSO_4 \cdot 7H_2O$, 40 mg $CaCl_2$, 100 mg K_2HPO_4, and 100 mg NH_4Cl or 100 mg NH_4NO_3 per liter. The medium was completed with 0.5 ml of a minor element solution (Whittier and Steeves, 1960), 8 ml of a FeEDTA solution (Sheat *et al.*, 1959) and 2 g of glucose. The medium was solidified with 1.0% agar and was at pH 5.5 after autoclaving. Any modifications to the basic nutrient medium are presented with the results.

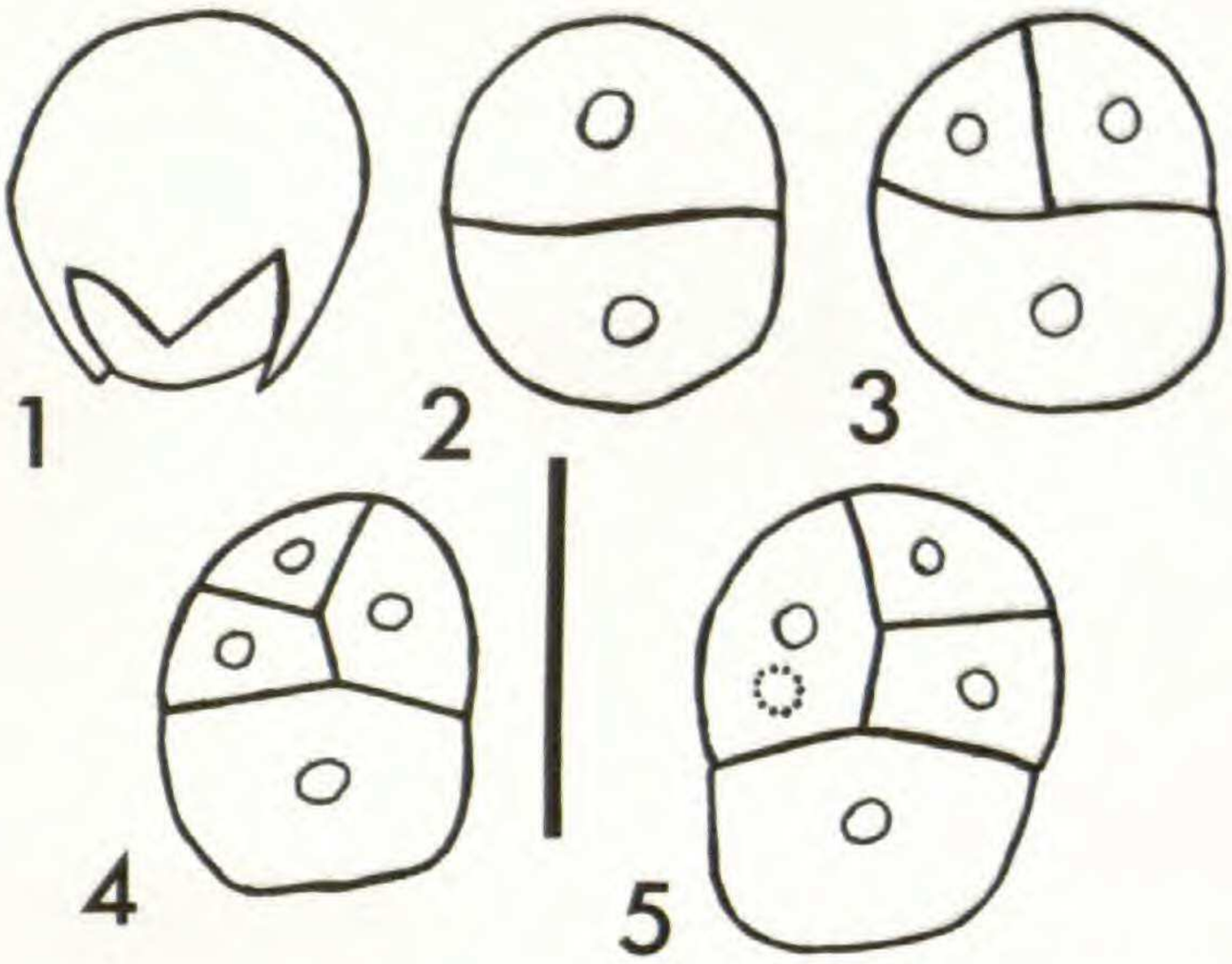
The sample size for calculating the average sizes of gametophytes or gametangia was 30. For determining the percentage of spore germination 500 or more spores were examined.

Early stages of gametophyte development were cleared and stained with acetocarmine-choral hydrate and drawn with a camera lucida for study (Whittier, 1981). Mucilage formation on the proximal cell and rhizoids was demonstrated by alcian blue staining (Whittier and Peterson, 1984). For the later developmental stages, the gametophytes were fixed with Randolph's Modified Navashin Fluid (CRAF) (Johansen, 1940). After fixation, the gametophytes were embedded in paraffin, and sectioned by conventional techniques (Johansen, 1940). The sections were stained with Heidenhain's hematoxylin, safranin O, and fast green.

For scanning electron microscopy, the gametophytes were fixed overnight on ice in a 1:1 solution of 4% glutaraldehyde and 10% acrolein in 0.1 mol/L Hepes buffer (pH 6.8) (Whittier and Peterson, 1984). The gametophytes were postfixed in 1% osmium tetroxide in 0.1 mol/L Hepes buffer (pH 6.8) at room temperature for 1 hour. They were then treated with 1% aqueous thiocarbohydrazide for 30 minutes after the osmium postfixation. The gametophytes were refixed with 2% osmium tetroxide in water for 1 hour and then dehydrated in a graded acetone series. All specimens were critical point dried and coated with gold-palladium before observing with a Hitachi 4500 or 370 scanning electron microscope at 10 or 15kV.

Observations

The earliest germination occurred 8 days after the cultures were placed in the dark. After 3 weeks in the dark 40% of the spores had germinated. With

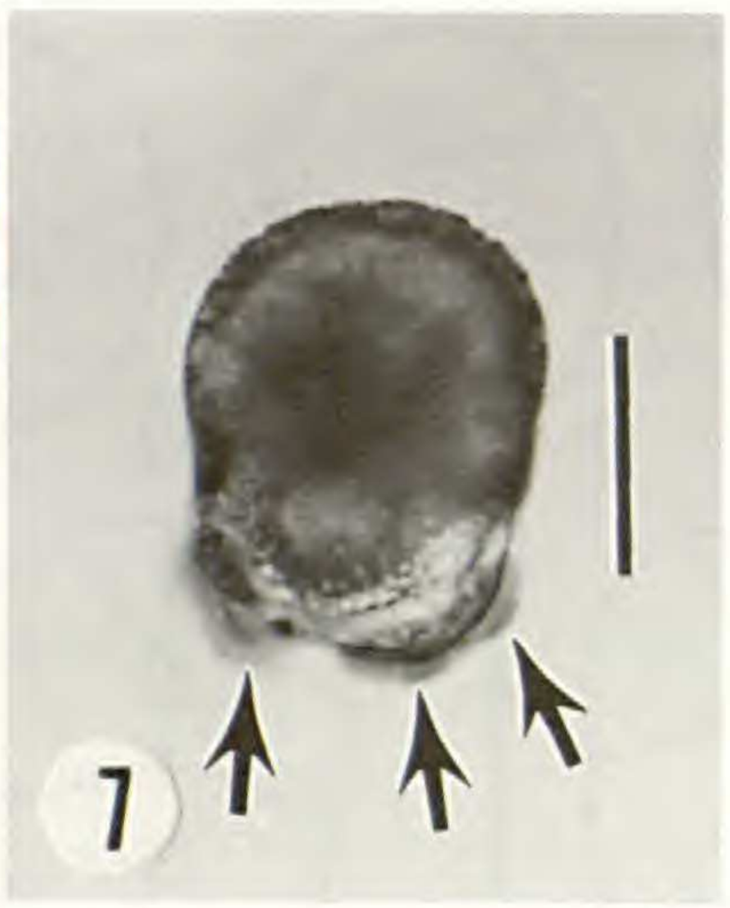


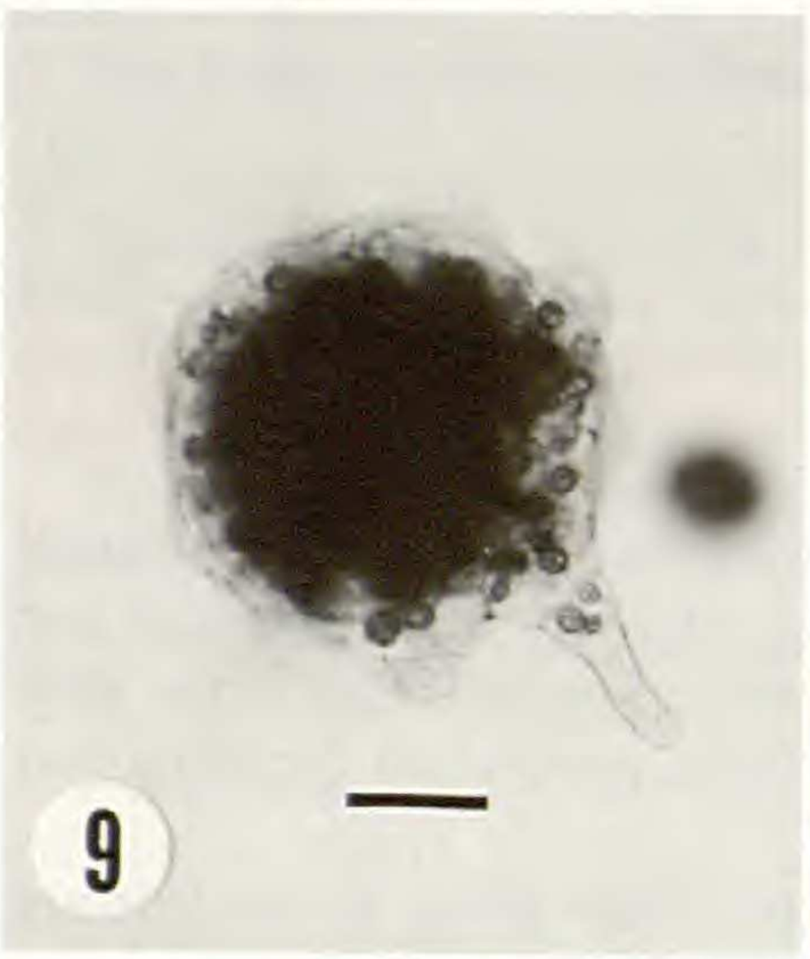
FIGS. 1–5. Early stages in gametophyte development of *Ophioglossum crotalophoroides*. The circles indicate nuclei and the dotted circle is a nucleus of the cell behind facing cell. 1. Germinating spore. 2. Two-celled gametophyte. 3. Three-celled gametophyte. 4. Four-celled gametophyte. 5. Five-celled gametophyte. Spore coats omitted in FIGS. 2–5. Bar = 50 μm.

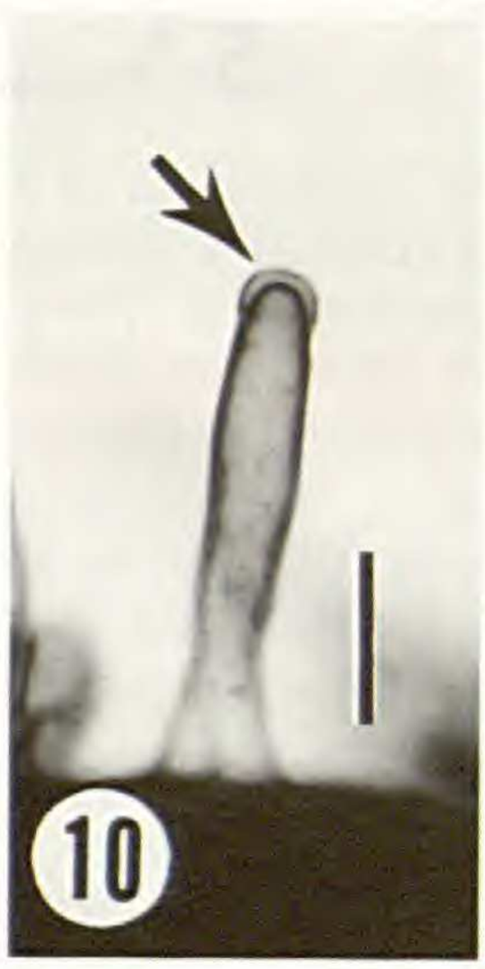
longer dark periods, up to 96% of the spores germinated. Spores maintained for a year in cultures that were illuminated for 14 of every 24 hours did not germinate. Shorter periods of illumination were also sufficient to stop germination. Daily exposures to 15 minutes of light prevented germination in a 28 day experiment.

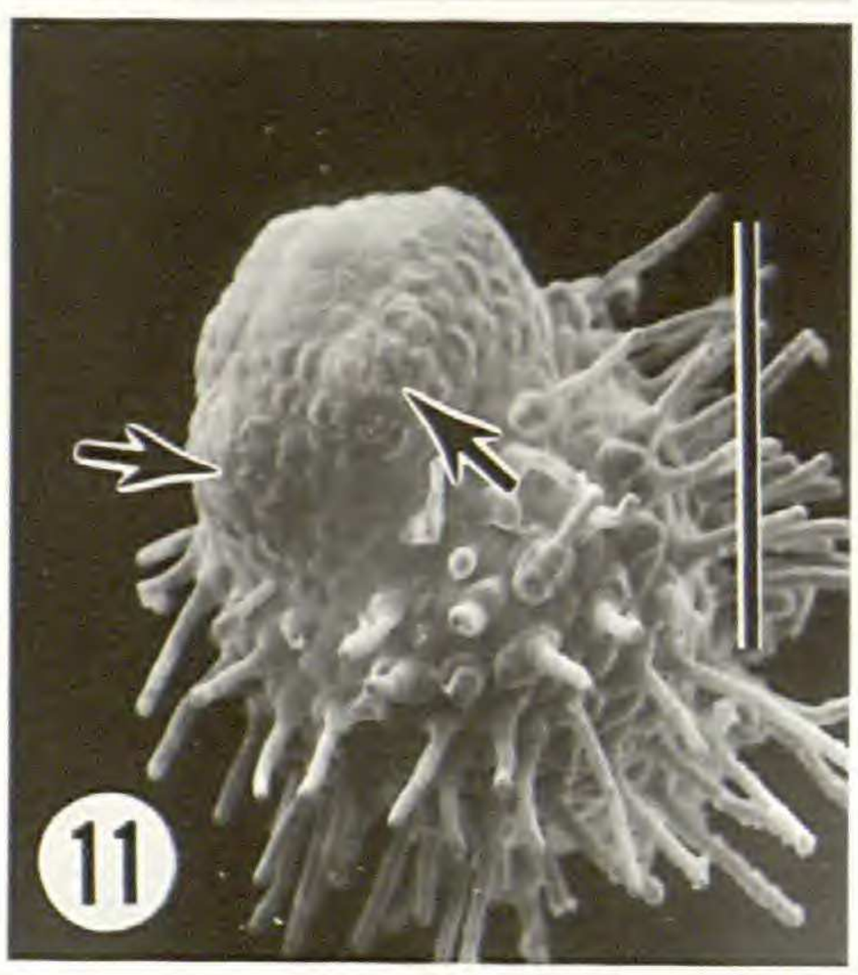
The spore coat cracked open at the triradiate ridge (Fig. 1) to initiate germination. Shortly after the spore coat ruptured, the spore divided perpendicular to its polar axis (Fig. 2). A proximal cell (near the triradiate ridge) and a distal cell (away from the triradiate ridge) were formed. As the two cells expanded the proximal cell bulged out of the spore coat forcing its lobes apart. The distal cell remained inside the spore coat and continued to divide. The second division was more or less parallel to the polar axis of the spore and divided the distal cell into two cells (Fig. 3). The third division, which was usually perpendicular to the polar axis of the spore, occurred in one of the two distal cells (Fig. 4). The fourth division occurred in the other of the two distal cells and its plane was usually perpendicular to the plane of the third division (Fig. 5). The divisions in the 5-celled and larger gametophytes were not followed because more variation existed in the later sequence of divisions and the shape of the young gametophytes made them difficult to follow.

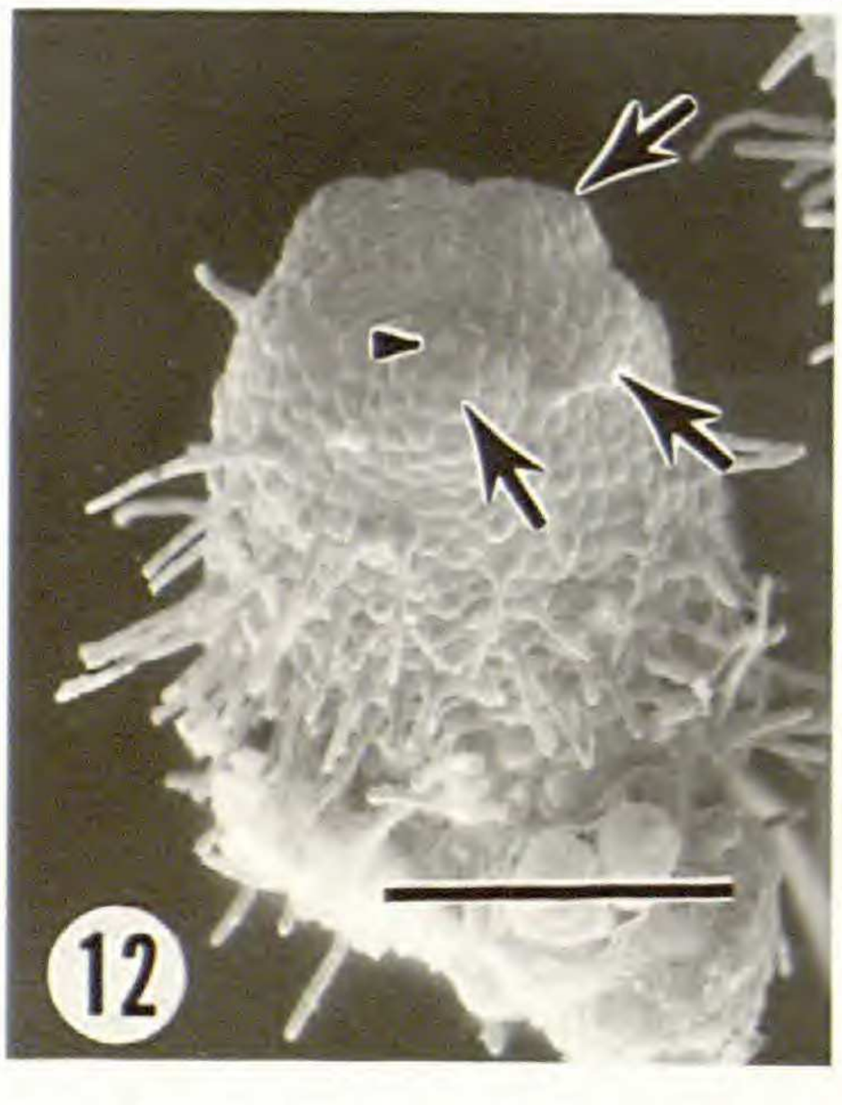
At about the 5-celled stage the proximal cell was fully extended beyond the spore coat (Fig. 6). Once this happened, mucilage, which stained for acid mucopolysaccharide with alcian blue, was secreted at the exposed end of this cell (Fig. 7). When fully secreted it took the shape of a triangular ring. The production of mucilage was not dependent on the availability of sugar because it formed at the same stage on gametophytes grown on a medium lacking sugar.

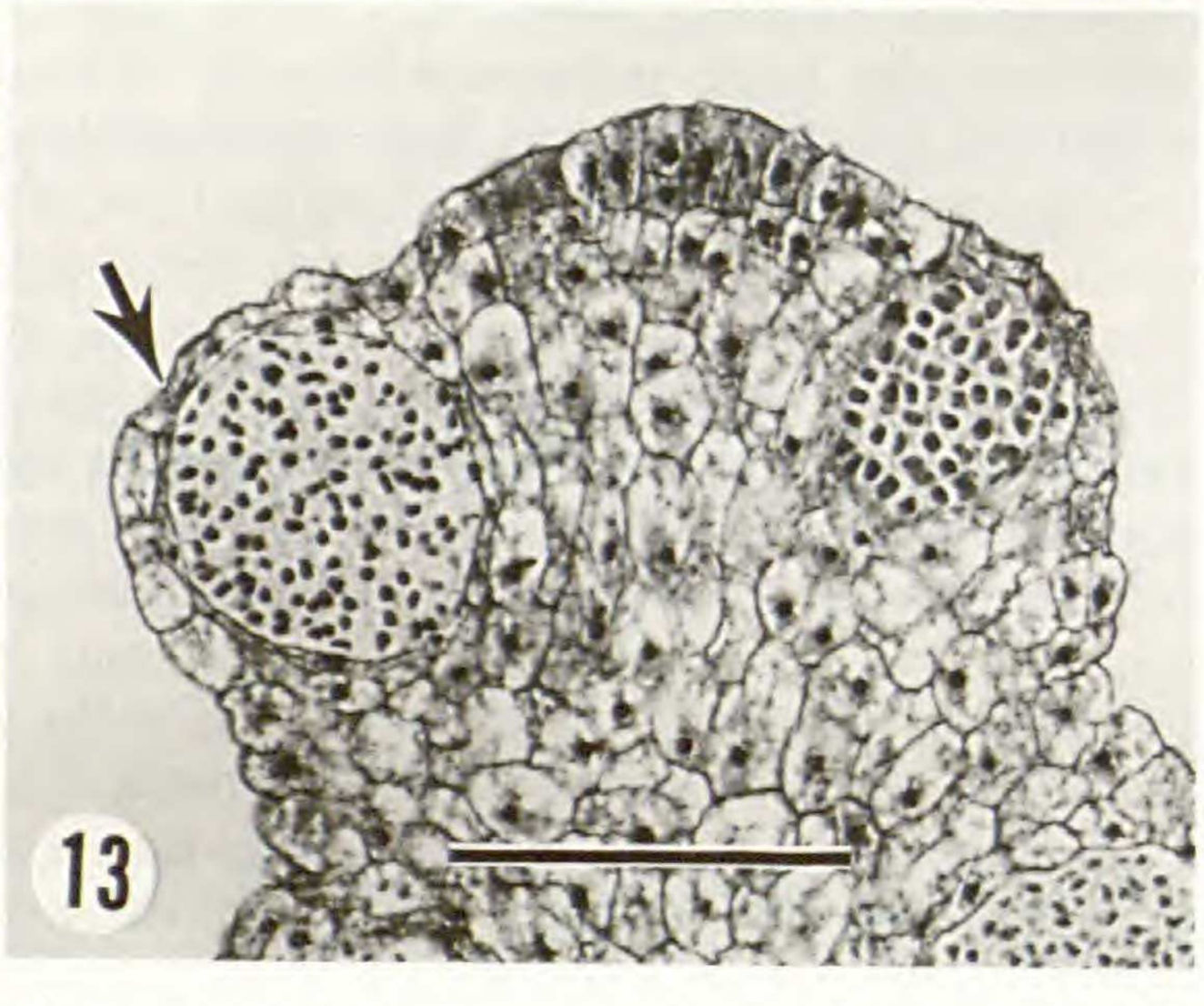












FIGS. 6–13. Gametophytes of *Ophioglossum crotalophoroides*. 6. Young gametophyte with exposed proximal cell, bar = 20 μm. 7. Mucilage (arrows) on proximal cell. Alcian blue staining. bar = 20 μm. 8. Spherical or globular gametophyte, bar = 50 μm. 9. Spherical or globular gametophyte with a rhizoid, bar = 50 μm. 10. Rhizoid with mucilage (arrow); alcian blue staining,

Shortly after the 5-celled stage, the gametophyte became free of the spore coat. With additional cell divisions, a small spherical or globular gametophyte was formed (Figs. 8, 9). It was at the spherical stage that rhizoids began to develop (Fig. 9). Regardless of the gametophyte age the rhizoids of *O. crotalophoroides* secreted mucilage that stains with alcian blue (Fig. 10).

The small globular gametophytes grew into short cylindrical gametophytes. At 100 days the gametophytes on the average were 0.3 mm long and 0.2 mm wide. By the time the gametophytes were 0.6 mm long their apical regions had expanded to a width of 0.4 mm. At this stage the gametophytes were conical or teardrop shaped (Figs. 11, 12, 14). The basal regions of these teardrop-shaped gametophytes had numerous rhizoids. The apical regions lacked rhizoids and the youngest of these gametophytes lacked gametangia.

Antheridia first formed 4.5 months after sowing the spores (Fig. 11). Gametophytes with 1–3 antheridia averaged 0.7 mm in length and 0.5 mm in width. The antheridia were almost completely sunken into the gametophyte tissue but are recognized at the surface by slightly raised areas (Figs. 11, 12). The antheridial jacket is two cells thick except at the opercular cell (Fig. 13). Although slightly longer than wide, the mass of sperm had almost a spherical shape. The average size of the sperm mass was 148 μm in length by 138 μm in width.

At 6.5 months the gametophytes began to form archegonia (Fig. 14). Gametophytes with 1–3 archegonia were on average 1.0 mm long and 0.7 mm wide. The archegonia had short necks with usually 2–3 tiers of neck cells exposed above the gametophyte surface (Fig. 15). Their average length from base of egg to tip of the neck was 160 μm (Fig. 16).

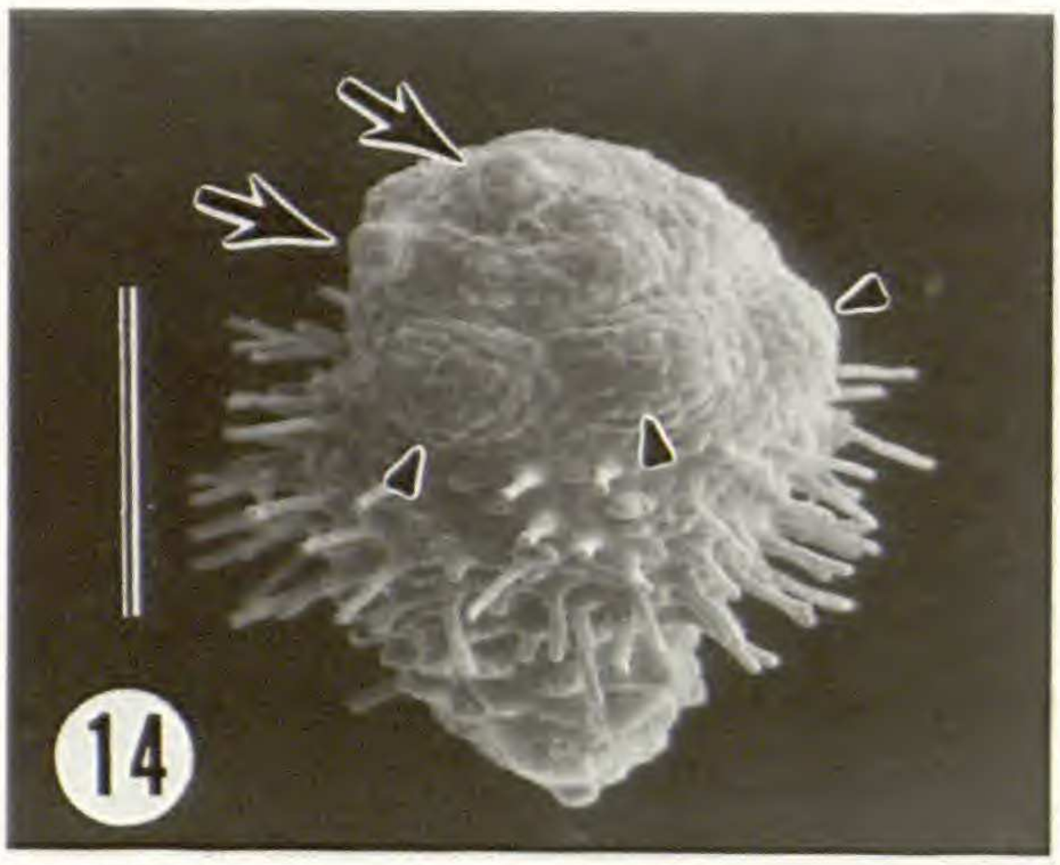
Once the gametophytes formed archegonia at 6.5 months they were sexually mature. Their gametangia are functional under these cultural conditions because sporophytes developed in cultures with moisture on the surface of the nutrient medium (Fig. 17). Functional fertile spikes did not form on these young sporophytes, however in some cases an abortive fertile spike was associated with the first leaf (Fig. 17, arrow).

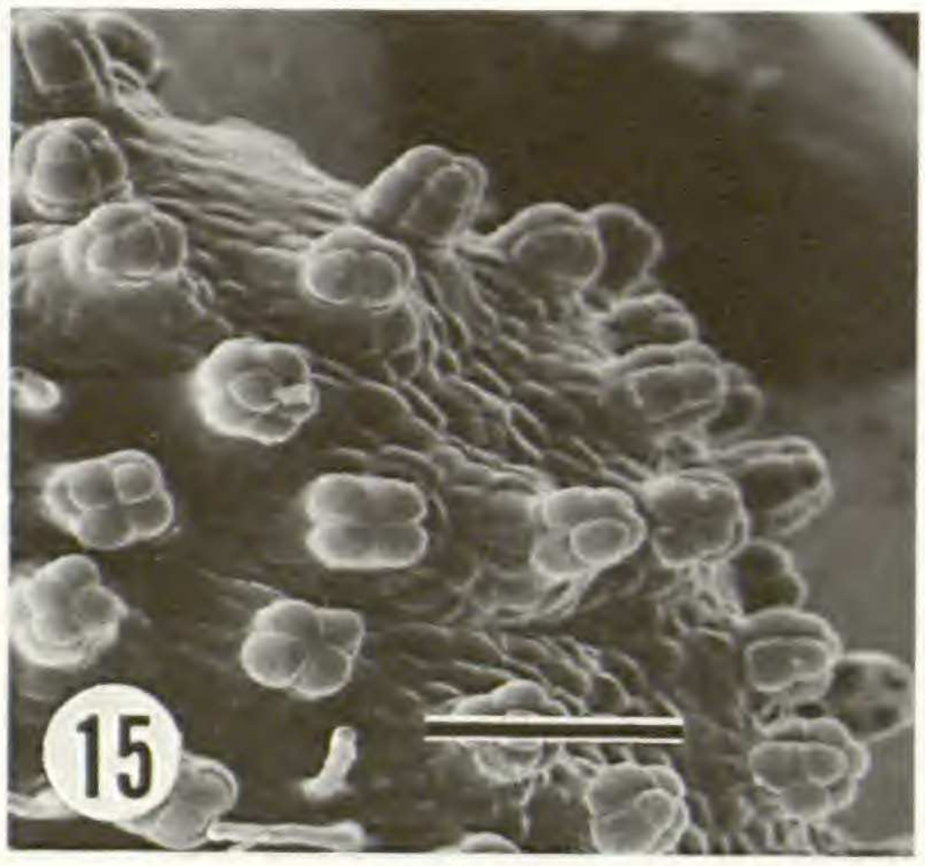
Discussion and Conclusions

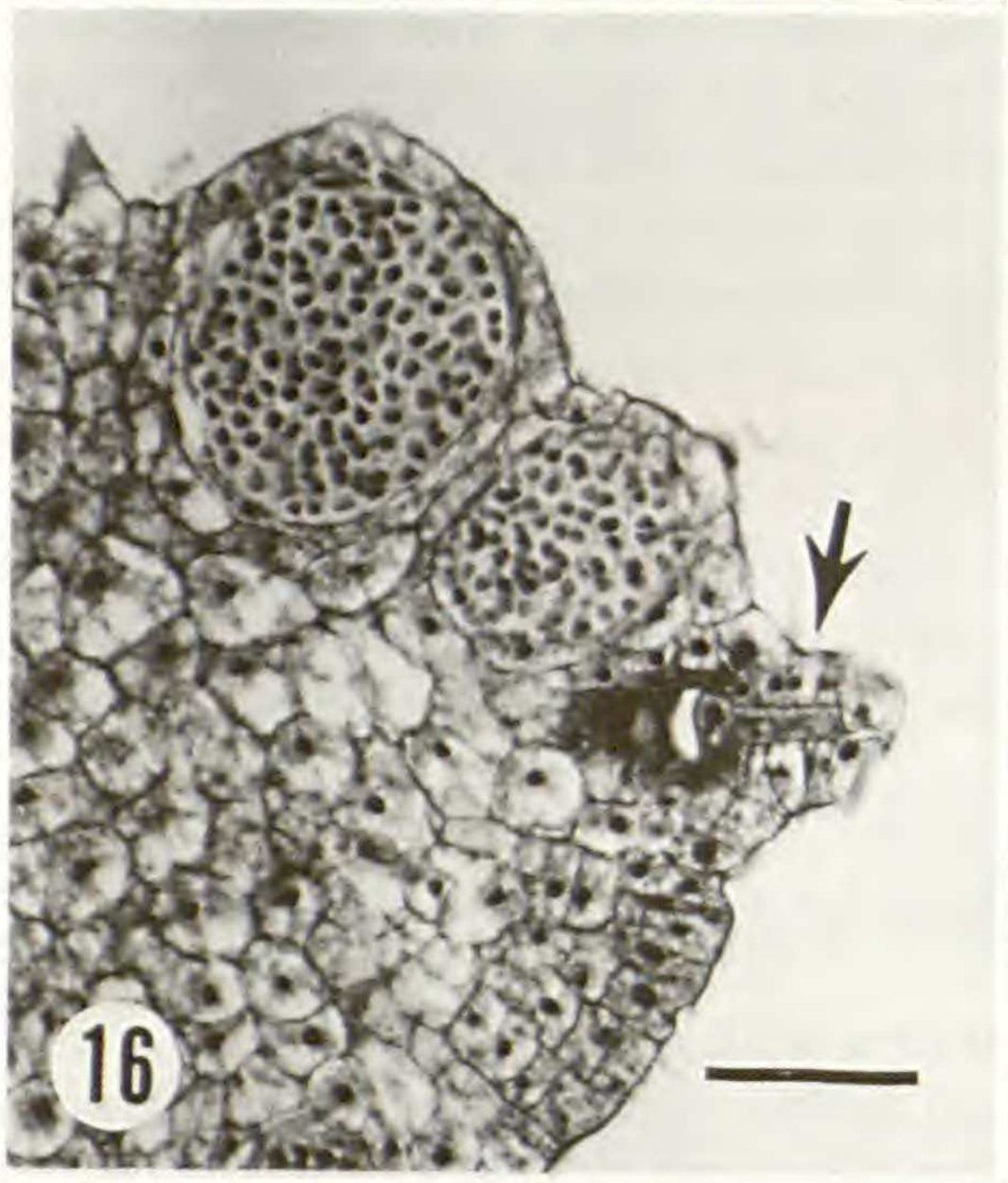
The average germination times for green pteridophyte spores and non-green fern spores are 1.5 and 9.5 days respectively (Lloyd and Klekowski, 1970). Spores of the Ophioglossaceae were not studied by Lloyd and Klekowski (1970). The average germination times of 54 and 37 days for spores of *Ophioglossum* (excluding *O. crotalophoroides*) and *Botrychium* respectively (Table 1) support the generalization that spore germination is slow for this

←

bar = 100 μm. 11. Young gametophyte with sunken antheridia (arrows), bar = 500 μm. 12. Young gametophyte with sunken antheridia (arrows), arrowhead indicates opercular cell, bar = 500 μm. 13. Longitudinal section through apical region of gametophyte with sunken antheridia, arrow indicates opercular cell of an antheridium, bar = 200 μm.







FIGS. 14–17. Gametophytes and young sporophyte of *Ophioglossum crotalophoroides*. 14. Gametophyte with two young archegonia (arrows) and sunken antheridia (arrowheads), bar = 500 μm. 15. Archegonia, bar = 250 μm. 16. Longitudinal section through apical region of gametophyte with an archegonium (arrow) and antheridia, bar = 100 μm. 17. Young sporophyte, arrow indicates abortive fertile spike, bar = 5 mm.

family. The germination of spores of *O. crotalophoroides* in 8 days is the second fastest germination reported for the Ophioglossaceae (Table 1). Compared with the average germination times for this family, the spores of *O. crotalophoroides* germinate rapidly. Spore germination in this species is also faster than the average germination time (9.5 days) for other non-green fern spores (Lloyd and Klekowski, 1970).

The pattern of cell divisions in the early development of the gametophytes of *O. crotalophoroides* is basically the same as reported for *Botrychium* and other species of *Ophioglossum* (Whittier, 1981). There was nothing unusual about

TABLE 1. Days to spore germination in the Ophioglossaceae.

Species	Days	Reference
Ophioglossum		
crotalophoroides[1]	8	Present study
engelmannii[1]	71	Whittier, umpubl.
intermedium[2]	51	Campbell, 1907
moluccanum[2]	3	Campbell, 1907
pendulum[2]	36	Campbell, 1907
pusillum[1]	90	Whittier, unpubl.
Botrychium		
biternatum[1]	28	Whittier, 1981
dissectum[1]	56	Whittier, 1981
gallicomontanum[1]	31	Whittier, unpubl.
jenmanii[1]	21	Whittier & Thomas, 1993
lanceolatum[1]	41	Whittier, unpubl.
lunarioides[1]	21	Whittier, 1981
matricariifolium[1]	56	Whittier, 1981
virginianum[1]	42	Whittier, unpubl.

[1] germination in axenic culture, [2] germination on wet humus.

the first 4–5 divisions after germination. The formation of mucilage on the exposed proximal cell of *O. crotalophoroides* appears normal for this family. It has been found previouly in species of *Botrychium* (Melan and Whittier, 1989). The production of mucilage on the rhizoids of *O. crotalophoroides* is typical for the Ophioglossaceae. Mucilage has been found on the rhizoids of *Botrychium* species examined from axenic culture (Whittier and Peterson, 1984). It has not been reported for other species of *Ophioglossum* because they did not develop rhizoids under culture conditions.

The gametangia that developed were similar to those found on gametophytes of *O. crotalophoroides* from soil. The antheridia were almost completely sunken with a bistratose jacket and, as reported by Mesler (1976), a single opercular cell. Archegonia on gametophytes from culture had short exposed necks that are similar to those on gametophytes from soil (Mesler, 1976). The gametangia on these gametophytes were normal for *Ophioglossum* (Pant *et al.*, 1984).

A major difference between the gametophytes of *O. crotalophoroides* from soil and culture is the absence of a mycorrhizal fungus in the cultured gametophytes. This is typical for normally mycorrhizal gametophytes growing in axenic culture. The sugar in the nutrient medium replaces the need for the mycorrhizal fungus as a carbon source. Whether the fungus under natural conditions supplies additional organic materials to the gametophyte is unknown at this time.

Gametophyte lengths at day 100 from sowing and at the times of early antheridia and archegonia formation provided a chance to determine average growth rates. The growing time was computed as the time from sowing minus the time to germination. Using these calculations the average growth in length

per day for gametophytes of *O. crotalophoroides* was 3.3 μm for the first 3 months after germination and 5.5 μm for 4.2 and 6.2 months after germination. These rates were faster than the 2.5 μm per day for gametophytes of *Botrychium virginianum* and *B. dissectum* forma *obliquum* for 4 months of growth after germination in culture (Whittier unpubl.).

The average length and width of gametophytes of *O. crotalophoroides* with 1–3 antheridia was 0.7 mm by 0.5 mm. The 10 smallest gametophytes from soil with only antheridia averaged 1.0 mm long by 0.7 mm wide for *B. dissectum* and 1.0 mm long by 0.8 mm wide for *B. virginianum* (Foster, 1964). The average sizes for *Botrychium* gametophytes with antheridia from soil are presented because they were smaller than gametophytes of these species from culture with 1–3 antheridia (Whittier unpubl.). The average size of the 10 smallest gametophytes of *B. dissectum* with archegonia from soil was 1.6 mm long by 1.2 mm wide and that of the 6 smallest gametophytes of *B. virginianum* with archegonia was 1.9 mm by 1.4 mm (Foster, 1964). These average sizes were again smaller than those for gametophytes of these species bearing 2–3 archegonia from culture (Whittier, unpubl.). The average sizes of the *Botrychium* gametophytes with archegonia are larger than those of *O. crotalophoroides* bearing 1–3 archegonia which averaged 1.0 mm long and 0.7 mm wide. These comparisons show that the gametophytes of *O. crotalophoroides* from culture develop gametangia at smaller sizes than either *Botrychium* species.

The time to sexual maturity for *O. crotalophoroides* at 6.5 months from sowing the spores is much faster than the 22 months reported for *B. dissectum* in culture (Whittier, 1972). Besides maturing faster than *B. dissectum*, these gametophytes mature much quicker than the perennial gametophytes studied by Bruchmann (1904), Campbell (1911), and Pant *et al.* (1984). The only gametophytes of the Ophioglossaceae that may mature as fast are possibly the annual gametophytes of *O. moluccanum* (Campbell, 1907). The rapid maturation of the gametophytes of *O. crotalophoroides* in axenic culture helps to confirm the report of rapid reproduction in this species by Mesler (1976).

The accelerated maturation of these gametophytes is promoted by each of the following factors. Quick spore germination initiated gametophyte development sooner. Rapid growth produced larger gametophytes in a shorter time. The formation of antheridia and archegonia on smaller gametophytes reduced the amount of growth necessary to attain maturity. Collectively, these conditions—rapid germination, rapid growth, and reduced amount of gametophyte tissue necessary for gametangia formation—bring about the accelerated sexual maturity of these gametophytes.

ACKNOWLEDGMENTS

I thank R. Dale Thomas at Northeast Louisiana University for assistance in obtaining the spores of *O. crotalophoroides*. The spores were supplied or were from plants at sites located by him. I also thank R. L. Peterson for use of his laboratory facilities at the University of Guelph (Canada) where the scanning electron microscopy was done.

Literature Cited

Boullard, B. 1963. Le gametophyte des Ophioglossacées. Considérations biologiques. Bull. Soc. Linn. Normandie 4:81–97.

Bruchmann, H. 1904. Ueber das Prothallium und die Keimpflanze von *Ophioglossum vulgatum* L. Bot. Zeitung, 2. Abt. 62:227–247.

Campbell, D. H. 1907. Studies on the Ophioglossaceae. Ann. Jardin Bot. Buitenzorg 6:138–194.

Campbell, D. H. 1911. *The Eusporangiatae*. Publ. Carnegie Inst. Wash. No. 140.

Foster, D. B. 1964. The gametophytes and embryogeny of five species of *Botrychium*. Ph.D. Thesis, Cornell University, Ithaca, New York.

Johansen, D. A. 1940. *Plant Microtechnique*. McGraw-Hill, New York.

Lloyd, R. M. and E. J. Klekowski Jr. 1970. Spore germination and viability in Pteridophyta: Evolutionary significance of chlorophyllous spores. Biotropica 2:129–137.

Melan, M. E. and D. P. Whittier. 1989. Characterization of mucilage on the proximal cells of young gametophytes of *Botrychium dissectum* (Ophioglossaceae). Amer. J. Bot. 76:1006–1014.

Mesler, M. R. 1976. Gametophytes and young sporophytes of *Ophioglossum crotalophoroides* Walt. Amer. J. Bot. 63:443–448.

Mesler, M. R., R. D. Thomas and J. G. Bruce. 1975. Mature gametophytes and young sporophytes of *Ophioglossum nudicaule*. Phytomorphology 25:156–166.

Nayar, B. K. and S. Kaur. 1971. Gametophytes of homosporous ferns. Bot. Rev. 37:295–396.

Nozu, Y. 1961. The gametophyte of *Helminthostachys zeylanica* and *Ophioglossum vulgatum*. Phytomorphology 11:199–206.

Pant, D. D., D. D. Nautiyal and D. R. Misra. 1984. Gametophytes of Ophioglossaceae. Phyta Mon. 1:1–111.

Raghavan, V. 1989. *Developmental Biology of Fern Gametophytes*, Cambridge University Press, Cambridge, U.K.

Sheat, D. E. G., B. H. Fletcher and H. E. Street. 1959. Studies on the growth of excised roots. VII. The growth of excised tomato roots supplied with various inorganic sources of nitrogen. New Phytologist 58:128–154.

Whittier, D. P. 1964. The effect of sucrose on apogamy in *Cyrtomium falcatum*. Amer. Fern J. 54:20–25.

Whittier, D. P. 1972. Gametophytes of *Botrychium dissectum* as grown in sterile culture. Bot. Gaz. 133:336–339.

Whittier, D. P. 1981. Spore germinaton and young gametophyte development of *Botrychium* and *Ophioglossum* in axenic culture. Amer. Fern J. 71:13–19.

Whittier, D. P. and R. L. Peterson. 1984. The gametophyte of *Botrychium lunarioides* and its mucilage coated rhizoids. Canad. J. Bot. 62:2854–2860.

Whittier, D. P. and T. A. Steeves. 1960. The induction of apogamy in the bracken fern. Canad. J. Bot. 38:925–930.

Whittier, D. P. and R. D. Thomas. 1993. Gametophytes and sporophytes of *Botrychium jenmanii* in axenic culture. Internat. J. Plant Sci. 154:68–74.